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Processing Pathway Deduced from the Structures of *N*-Glycans in *Carica papaya*[1]

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A processing The processing pathway of *N*-glycans in *Carica papaya* was deduced from the structures of *N*-glycans. The *N*-glycans were liberated by hydrazinolysis followed by *N*-acetylation. Their reducing-end sugar residues were tagged with 2-aminopyridine and the pyridylamino (PA-) sugar chains thus obtained were purified by HPLC. Eleven PA-sugar chains were found, and their structures were analyzed by two-dimensional sugar mapping combined with partial acid hydrolysis and exoglycosidase digestion. The structures of the *N*-glycans were of the highmannose types with xylose and fucose; however, among them two new *N*-glycans, Manα1-6(Manα1-3)Manα1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc and Manα1-3Manα1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc, were found. Judging from these structures together with Manα1-6(Manα1-3)Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc reported previously [Shimazaki, A., Makino, Y., Omichi, K., Odani, S., and Hase, S. (1999) *J. Biochem.* 125, 560–565], a processing pathway for *N*-glycans in *C. papaya* is inferred in which the activity of Golgi α-mannosidase II is incomplete.

Key words: *Carica papaya*, *N*-glycan, Golgi α-mannosidase II, processing pathway, pyridylamination.

The processing pathway of *N*-glycans in plant glycoproteins has been reported (*1*, *2*). This pathway starts from the *en bloc* transfer of $Glc_3Man_9GlcNAc_2$ to nascent polypeptide chaints in the endoplasmic reticulum. The processing of $Glc_3Man_9GlcNAc_2$ begins with the removal of three glucose and four mannose residues through sequential digestions with α-glucosidases and α-mannosidases. The product of these digestions, $Man_5GlcNAc_2$, is the substrate for *N*-acetylglucosaminyltransferase I, which produces the hybrid type *N*-glycan $GlcNAcMan_5GlcNAc_2$. Various complex-type and xylomannose-type *N*-glycans found in plant glycoproteins are produced from $GlcNAcMan_5GlcNAc_2$ on removal of two mannose residues through the action of Golgi α-mannosidase II. Recently, a new xylomannose-type *N*-glycan, Manα1-6(Manα1-3)Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc, was found in the proteinase inhibitor isolated from the latex of *Carica papaya* (*3*, *4*). This structure is not an intermediate in the processing pathway described above. To clarify this point further, the authors analyzed the whole structures of the *N*-glycans in *Carica papaya* and infer a processing pathway for the *N*-glycans on the basis of the structures identified.

MATERIALS AND METHODS

Materials—Fresh leaves of *C. papaya* were a gift from Higashiyama Botanical Garden (Nagoya). Latex was prepared from the leaves. PA-Fuc, PA-Gal, PA-GalNAc, PA-Glc, PA-GlcNAc, PA-Man, PA-Xyl, and PA-isomaltose were previously reported (*5*). PA-isomaltooligosaccharides were purchased from Takara Biomedicals (Kyoto). Sugar Chains 1–8, 10, 11, 13, and 15–21 were prepared as reported (*6*). Sugar Chain 14 was prepared from the proteinase inhibitor from papaya latex as described previously (*4*) (Table I). Jack bean α-mannosidase and *Chronia lampus* α-fucosidase were from Seikagaku Kogyo (Tokyo).

A Capcell Pak C8-UG120 column (4.6 × 150 mm) was purchased from Shiseido (Tokyo), a Q-Sepharose column (16 × 80 mm) from Pharmacia (Uppsala, Sweden), a Cosmosil 5C18-P column (1.5 × 250 mm) from Nacalai Tesque (Kyoto), Shodex Asahipak NH2P-50 columns (4.6 × 50 mm and 4.6 × 250 mm) from Showa Denko (Tokyo), Dowex 50W-X2 (200–400 mesh) from Dow Chemicals (Richmond, VA), and a TSK-gel Sugar AXI column (4.6 × 150 mm) and TSK-gel HW-40F from Tosoh (Tokyo).

High-Performance Liquid Chromatography of PA-Sugar Chains—Reversed-phase HPLC was performed on a Cosmosil 5C18-P column at the flow rate of 150 μl/min at 25°C. The column was equilibrated with 20 mM ammonium ace-

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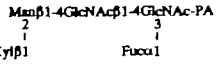
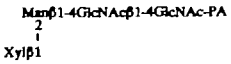
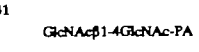
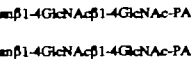
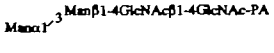
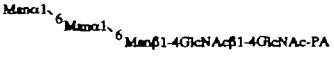
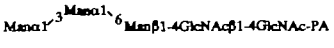
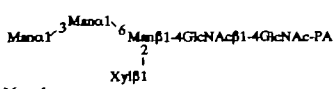
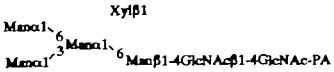
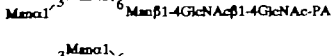
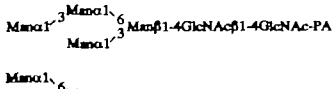
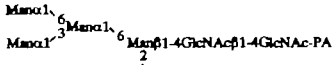
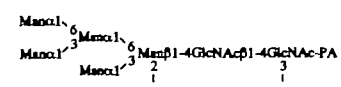
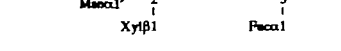
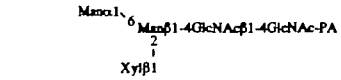
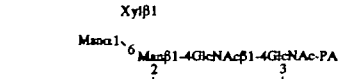
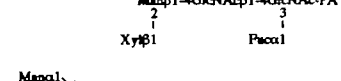
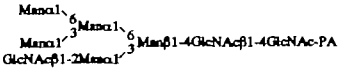
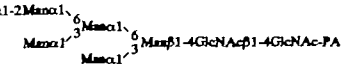
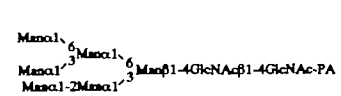
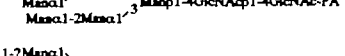
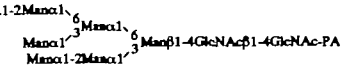
Abbreviations: *R*-value, reversed-phase value; PA-, pyridylamino. The structures and abbreviations of the sugar chains used are listed in Table I. Enzymes: α-mannosidase [EC 3.2.1.24], α-L-fucosidase [EC 3.2.1.51].

TABLE I. **Structures and designations of pyridylaminated sugar chains used in this study.**

Structure	Sugar Chain	HPLC	
		Reversed-phase[a]	Size-fractionation[b]
	1	30.6	3.7
	2	40.8	2.9
	3	26.7	1.7
	4	33.4	2.6
	5	33.4	3.5
	6	41.7	3.7
	7	41.6	4.5
	8	42.0	4.3
	9	(46.4)[c]	(4.7)
	10	43.0	5.3
	11	43.4	5.4
	12	(46.3)	(5.7)
	13	43.9	6.4

[a]The reversed-phase scale and data are from Ref. *6* except for Sugar Chain 14. [b]Glucose units. [c]Figures in parentheses are calculated values, as reported previously (*6*, *14*).

Structure	Sugar Chain	HPLC	
		Reversed-phase[a]	Size-fractionation[b]
	14	30.9	7.9
	15	46.1	4.0
	16	36.6	4.6
	17	42.3	7.1
	18	37.8	7.4
	19	40.2	7.4
	20	33.1	8.4
	21	35.8	10.1
	22	-	-
	23	(41.6)	(6.0)
	24	(41.3)	(6.2)

tate buffer, pH 4.0, containing 0.075% 1-butanol. After injecting a sample, the concentration of 1-butanol was increased linearly to 0.4% in 90 min. The elution was monitored by measuring the fluorescence (excitation wavelength, 315 nm; emission wavelength, 400 nm). The elution positions of the PA-sugar chains are expressed as *R*-values (reversed-phase scale), as previously reported (*6*).

Size-fractionation HPLC was performed on a Shodex Asahipak NH2P-50 column (4.6 × 50 mm) at the flow rate of 0.6 ml/min at 25°C using two eluents. Eluent A was 0.3% (v/v) acetic acid in a 1:4 (v/v) mixture of acetonitrile:water adjusted to pH 7.0 with aqueous ammonia; Eluent B was 0.3% (v/v) acetic acid in a 93:7 (v/v) mixture of acetonitrile:water adjusted to pH 7.0 with aqueous ammonia. The column was equilibrated with 3% Eluent A. After injecting a sample, the proportion of Eluent A was increased linearly to 33% in 1 min, and then to 71% in 34 min. The elution positions of the PA-sugar chains are expressed as glucose units with PA-isomaltooligosaccharides.

Size-fractionation HPLC was also performed on a Shodex Asahipak NH2P-50 column (4.6 × 250 mm) at 25°C at the flow rate of 0.8 ml/min. Two eluents were used, A and C. Eluent A was the same as described above. Eluent C was acetonitrile:water:acetic acid (900:100:3, v/v/v) titrated to pH 7.0 with aqueous ammonia. The column was equilibrated with 5% Eluent A. After injecting a sample, linear gradient elutions was performed to 14% Eluent A in 3 min, to 25% in 17 min, to 50% in 60 min, and to 75% in 5 min.

PA-monosaccharides were analyzed by Sugar AXI HPLC as already reported (*7*).

Analysis of Oligosaccharide Structures from the Reducing End Terminal—The procedure was carried out according to the reported method (*8*). Briefly, 200 μl of a 1 M trifluoroacetic acid aqueous solution was added to 100 pmol of a PA-sugar chain. Half of the resultant mixture was hydrolyzed at 100°C for 10 min and the other half for 1 h. The hydrolysates were then combined, freeze-dried, and *N*-acetylated as reported (*8*). The sample obtained was size-fractionated every 1 glucose unit using PA-isomaltooligosaccharides as the elution scale. After lyophilization, a part of each fraction was analyzed by reversed-phase HPLC.

Preparation of PA-Sugar Chains—Sugar chains were

released from 50 mg of finely powdered freeze-dried leaves by hydrazinolysis (10 ml, 100°C for 10 h) followed by *N*-acetylation, and the reducing ends of the sugar chains liberated were pyridylaminated as described previously (*9*, *10*). The pH of the reaction mixture was brought to 10 with 6 M aqueous ammonia, and then excess reagents were extracted five times with an equal volume of chloroform. After pH adjustment to 6.0 with acetic acid, the water phase was concentrated to a small volume, and then the solution was chromatographed on an HW-40F column (1.2 × 45 cm) using 0.01 M ammonium acetate buffer, pH 6.0. The fraction eluted between the void volume and 40 ml was collected as a PA-sugar chain fraction.

Enzymatic Hydrolysis of PA-Sugar Chains—A PA-sugar chain (100–200 pmol) was digested with 300 milliunits of α-mannosidase in 30 μl of 50 mM sodium citrate buffer, pH 4.6, at 37°C for 6 h; with 5 milliunits of α-fucosidase in 50 μl of 100 mM ammonium acetate buffer, pH 3.9, at 37°C for 6 h; or with 30 μl of papaya latex at 37°C for 1–40 h. The enzymatic reactions were terminated by heating at 100°C for 3 min.

RESULTS AND DISCUSSION

Preparation and Separation of PA-Sugar Chains from Papaya Leaves—Whole PA-sugar chains were prepared from 50 mg of freeze-dried papaya leaves as described under "MATERIALS AND METHODS". The PA-sugar chain fraction obtained was separated by size-fractionation HPLC (Fig. 1), Fractions A–H being collected. Fractions D and E were further separated by reversed-phase HPLC (Fig. 2), Fractions Da, Db, and Ea–Ec being collected. All

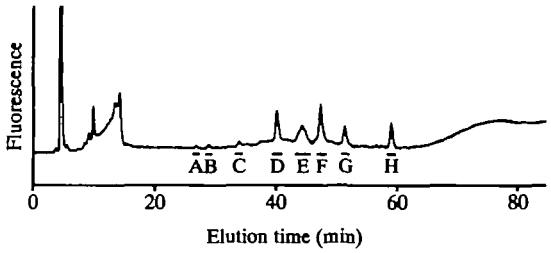


Fig. 1. **Size-fractionation HPLC of PA-oligosaccharides from leaves of *C. papaya*.** HPLC was preformed on a Shodex Asahipak NH2P-50 column (0.46 × 25 cm). Fractions A–H were collected, as indicated by the bars. The peaks appearing between 5 and 15 min are due to contaminating materials.

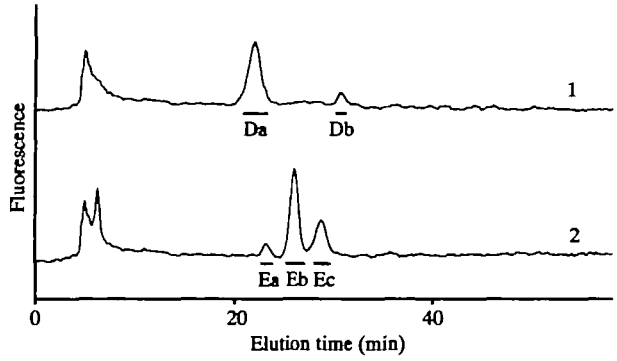


Fig. 2. **Reversed-phase HPLC of Fractions D (1) and E (2).** The fractions were pooled as indicated by the bars. The peaks appearing at around 6 min are due to contaminating materials.

TABLE II. **HPLC analysis of sequential glycosidase digests of Fractions A–H, and their reducing ends.**

Fraction	Successive treatment with glycosidases	HPLC		Sugar chain indentified[c]	Reducing-end PA-sugar
		Reversed-phase[a]	Size-fractionation[b]		
A	No treatment	30.1	3.7	1[d]	PA-GlcNAc
	α-L-Fucosidase	39.9	2.9	2	
B	No treatment	36.0	4.6	16[d]	PA-GlcNAc
	α-Mannosidase	30.3	3.7	1	
	α-L-Fucosidase	40.2	2.9	2	
C	No treatment	38.0	5.4	—	PA-GlcNAc
	α-Mannosidase	30.6	3.7	1	
	α-L-Fucosidase	40.9	2.9	2	
Da	No treatment	30.5	6.5	—	PA-GlcNAc
	α-Mannosidase	30.6	3.7	1	
	α-L-Fucosidase	40.9	2.9	2	
Db	No treatment	43.6	6.4	13[d]	PA-GlcNAc
	α-Mannosidase	33.5	2.6	4	
Ea	No treatment	38.2	7.4	18[d]	PA-GlcNAc
	α-Mannosidase	33.0	2.6	4	
Eb	No treatment	40.2	7.4	19[d]	PA-GlcNAc
	α-Mannosidase	33.9	2.6	4	
Ec	No treatment	42.2	7.1	17[d]	PA-GlcNAc
	β-*N*-Acetylhexosaminidase	43.5	6.4	13	
	α-Mannosidase	33.8	2.6	4	
F	No treatment	30.8	7.9	14[d]	PA-GlcNAc
	α-Mannosidase	29.9	3.7	1	
	α-L-Fucosidase	41.0	2.9	2	
G	No treatment	32.5	8.4	20[d]	PA-GlcNAc
	α-Mannosidase	34.1	2.6	4	
H	No treatment	35.0	10.2	21[d]	PA-GlcNAc
	α-Mannosidase	33.9	2.7	4	

[a]Reversed-phase scale (*6*). [b]Glucose units. [c]The numbers refer to the sugar chain structures shown in Table I. [d]The elution positions were confirmed by coelution with the standard PA-sugar chains.

the fractions thus obtained gave a single peak when analyzed by reversed-phase HPLC (data not shown).

Structural Analysis of Fractions A–H Except for C and Da—As Fractions A–H, apart from C and Da, were eluted at the positions of standard PA-sugar chains, their structures were analyzed by exoglycosidase digestion combined with two-dimensional HPLC mapping (Table II). The structures are summarized in Fig. 3.

Structural Analysis of Fraction Da—Although Fraction Da was not identified as an authentic PA-sugar chain on the two-dimensional sugar map, the α-mannosidase digest was eluted at the position of Sugar Chain 1 (Table II). This digestion reduced the molecular size from 6.5 to 3.7 glucose units, indicating the removal of three α-mannose residues. Further digestion of the product with α-fucosidase resulted in the appearance of a new peak at the position of Sugar Chain 2. These results suggested that the structure of Fraction Da was Man_3(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)-GlcNAc-PA. The structure of the Man_3 part of Fraction Da was analyzed by a combination of partial acid hydrolysis and two-dimensional sugar mapping (*8*). The result of size-fractionation HPLC of the partial acid hydrolysates is shown in Fig. 4, Fractions Da1–7 being collected. Only PA-GlcNAc was detected in Fraction Da1 when it was analyzed on a TSK-gel Sugar AXI column (data not shown). Other fractions were analyzed by reversed-phase HPLC (Fig. 5). Sugar Chains 3 and 4 were detected in Fractions Da2 and Da3, respectively. In Fraction Da4, Sugar Chain 6 was detected, but Sugar Chain 5 was not. As Sugar Chain 5 appeared when Sugar Chain 14 was analyzed in the same way (*4*), Fraction Da had a Manα1-6Manβ, but not a Manα1-3Manβ, branch. Sugar Chains 7 and/or 8 were detected in Fraction Da5, and Sugar Chain 10 in Fraction Da6. From these results, the Man_3Manβ structure was considered to be Manα1-6(Manα1-3)Manα1-6Manβ. The detection of a peak at the position of Sugar Chain 12 was compatible with a Manα1-6(Manα1-3)Manα1-6Manβ structure (Fig. 5-5). The absence of a peak at the elution position of Fraction Da (Fig. 5-6) indicated that this fraction had an acid-labile substituent such as a fucose or sialic acid residue (*8*). The difference between the elution position of Fraction Da and that of the Sugar Chain 12 peak is characteristic of a Fucα1-3 residue (*6*). On the basis of these results, the structure of Fraction Da was deduced to be that shown in Fig. 3. To our knowledge, this structure has not hitherto been reported.

Structural Analysis of Fraction C—Fraction C was not identified as an authentic PA-sugar chain on the two-dimensional sugar map, but the α-mannosidase digest of Fraction C was eluted at the position of Sugar Chain 1 (Table II). This digestion reduced the molecular size from 5.4 to 3.7 glucose units, indicating the removal of two α-mannose residues. Further digestion of the product with α-fucosidase resulted in the appearance of a new peak at the position of Sugar Chain 2 (Table II). These results suggested that the structure of Fraction C was Man_2(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA. The structure of the Man_2 part of Fraction C was analyzed in the same way as described for Fraction Da. Fractions C2–C6 were obtained by size-fractionation HPLC, and Sugar Chains 3 and 4 were detected in Fractions C2 and C3, respectively (Fig. 6). In Fraction C4, Sugar Chain 6 was detected, but Sugar Chain 5 was not—as was the case with Fraction Da4. The peak indicated by bar K in Fig. 6-4 was subjected to HPLC on a Shodex Asahipak NH2P-50 column (0.46 ×

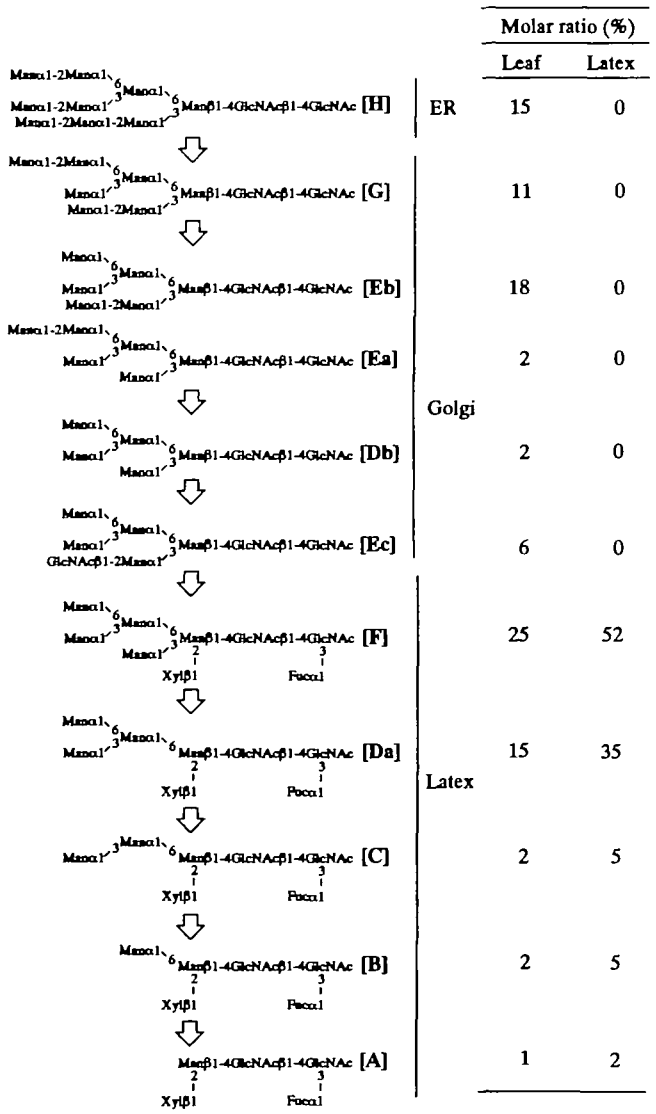


Fig. 3. **Structures and proposed processing pathway of *N*-glycans in *C. papaya*.**

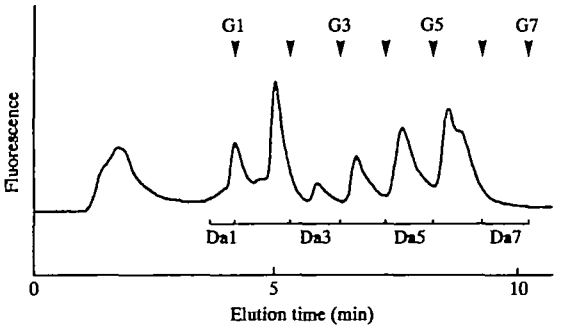


Fig. 4. **Size-fractionation HPLC of partial acid hydrolysates of Fraction Da.** Arrowheads G1–G7 indicate the elution positions of PA-glucose–PA-isomaltoheptaose, respectively. Fractions were collected as indicated by bars, Da1–Da7. The peak appearing at around 2 min is due to contaminating materials.

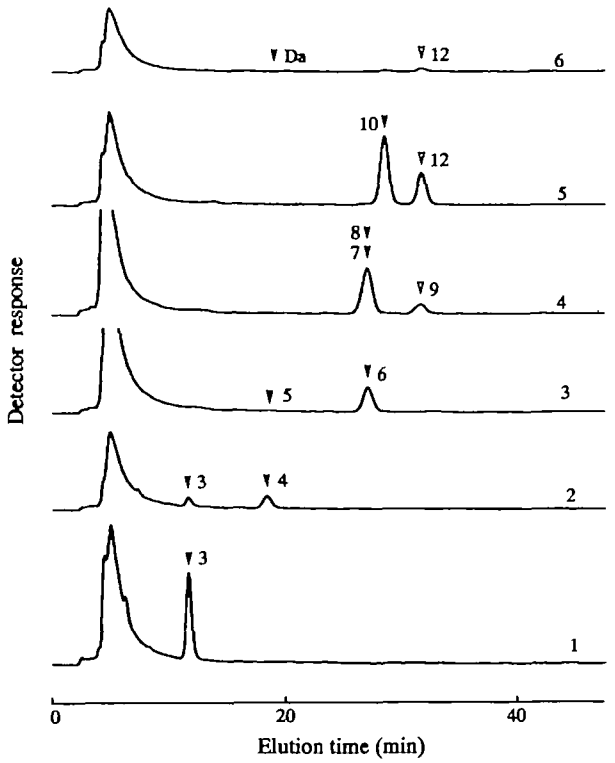


Fig. 5. **Reversed-phase HPLC analysis of Fractions Da2–Da7.** Elution profiles: 1, Fraction Da2; 2, Da3; 3, Da4; 4, Da5; 5, Da6; 6, Da7. Black and white arrowheads, respectively, indicate the elution positions of authentic PA-sugar chains, and the calculated elution positions of non-standard PA-sugar chain reported previously (*6*, *14*). Arrowhead Da indicates the elution position of Fraction Da; the other arrowheads correspond to the sugar chain numbers shown in Table I. The peaks appearing at around 5 min are due to contaminating materials such as plasticizers from the NH2P-50 resin.

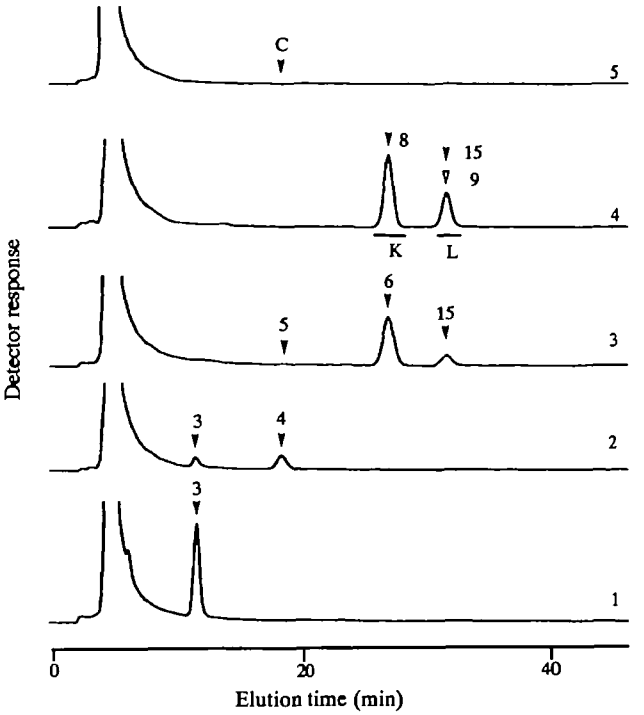


Fig. 6. **Reversed-phase HLPC analysis of Fractions C2–C6.** Elution profiles: 1, Fraction C2; 2, C3; 3, C4; 4, C5; 5, C6. Black and white arrowheads, respectively, indicate the elution positions of authentic PA-sugar chains, and the calculated elution positions of non-standard PA-sugar chain reported previously (*6*, *14*). Arrowhead C indicates the elution position of Fraction C; the other arrowhead numbers correspond to the sugar chain numbers shown Table I. The peaks appearing at around 5 min are due to contaminating materials such as plasticizers from the NH2P-50 resin.

25 cm) under the conditions with which Sugar Chains 7 and 8 were well separated. The result indicated that Fraction K actually consisted entirely of Sugar Chain 8 (data not shown). Size-fractionation HPLC of Fraction L on an Asahipak NH2P-50 column (0.46 × 25 cm) revealed that it was composed of Sugar Chains 9 and 15 in a ratio of 7:3. From these results, the $Man_2Man\beta$ structure was considered to be Manα1-3Manα1-6Manβ. The absence of a peak at the elution position of Fraction C (Fig. 6-5), and the difference between the elution position of Fraction C and that of the Sugar Chain 9 peak suggested a Fucα1-3 residue. On the basis of these results, the structure of Fraction C appeared to be that shown in Fig. 3. Again, to our knowledge this is a structure that has not previously been reported.

Preparation and Structural Analysis of PA-Sugar Chains from Papaya Latex—Whole PA-sugar chains of latex were prepared from 50 mg of freeze-dried papaya latex as described under "MATERIALS AND METHODS." The PA-sugar chains thus obtained were separated by size-fractionation and reversed-phase HPLC (data not shown). Five PA-sugar chains were obtained, and their structures were analyzed by partial acid hydrolysis combined with two-dimensional HPLC mapping (data not shown). The structures are also summarized in Fig. 3.

Proposed Hydrolysis Pathway of N-Linked Sugar Chains in C. papaya—Glycosidases and glycosyltransferases that catalyze the stepwise trimming and addition of sugar residues are generally considered to act in a co-ordinated and highly ordered fashion to form mature *N*-glycans in accordance with the reported processing pathway of plant glycoproteins (*1*, *2*). The proposed main hydrolysis pathway for *N*-glycans of *C. papaya*, as deduced from the *N*-glycan structures identified in this study, is shown in Fig. 3. Xylosyltransferase and fucosyltransferase transfer xylose and fucose, respectively, to the $GlcNAcMan_3GlcNAc_2$ structure in the reported processing pathway (*1*, *2*). Xylosyltransferase, however, can also transfer xylose to the $GlcNAcMan_5GlcNAc_2$ structure, though the reaction speed is lower (*1*, *11*). Sugar Chain 22, the product of the above reactions, was not detected in the present study, which may be due to the strong enzyme activity of β-*N*-acetylglucosaminidase present in the latex of *C. papaya* (*12*), as Sugar Chain 17 was easily hydrolyzed to sugar chain 13 with the latex (data not shown). The detection of Fractions F, Da, and C, together with the absence of Manα1-3(Manα1-6)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc, may indicate that the activity of Golgi α-mannosidase II is incomplete in *C. papaya*, as has been inferred in the case of *Nicotiana alata* (*2*, *13*). Structures Da, C, B, and A are considered to be the hydrolysis products derived from Structure F, as these sugar chains were obtained on digestion of Fraction F with the latex (data not shown).

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